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Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis

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Abstract

A PCR assay for the amplification of small subunit ribosomal DNA (SSU rDNA) of *Euryarchaea* was developed and used to detect archaeal rDNA in 37 (77%) out of 48 pooled subgingival plaque samples from 48 patients suffering from periodontal disease. One major group of cloned periodontal sequences was identical to *Methanobrevibacter oralis* and a second minor group to *Methanobrevibacter smithii*. These two groups and a third novel group were found to be more than 98% similar to each other over an 0.65-kb segment of the 16S rRNA gene sequenced. *M. oralis* was found to be the predominant archaeon in the subgingival dental plaque. Phylogenetic analysis of partial SSU rDNA sequences revealed evidence for a distinct cluster for human and animal *Methanobrevibacter* sp. within the *Methanobacteriaceae* family.

Keywords: Dental plaque; 16S rDNA; Archaeon; *Methanobacteriaceae*

1. Introduction

Methanogenic bacteria are found in various anoxic habitats, where biopolymers are degraded anaerobically [1,2]. Methanogens have been found in human and animal intestinal tract contents [3,4]. Methanogenesis was also detected in dental plaque of a monkey [5]. Previously, three studies reported methane production in enrichment cultures from human dental plaque. Methanogenesis occurred in subgingival plaque from 10 out of 36 [6] and from three out of 10 [7] periodontitis patients and in dental plaque from nine out of 20 healthy individuals [8]. Enriched bacteria were assigned to the genus *Methanobrevibacter* by phenotypic and immunological methods [6,7] and an oral isolate was described as *Methanobrevibacter oralis* [9].

Periodontal disease is a chronic inflammatory disease associated with a complex microflora consisting of several hundred mainly strictly anaerobic bacteria [10]. The prevalence and frequency of methanogenic bacteria in dental plaque of patients with different periodontal conditions could be examined by retrieving their rRNA or rDNA directly from the plaque sample by the polymerase chain reaction (PCR). The application of these methods to the subgingival plaque of a periodontitis patient with spirochete-specific PCR primers revealed a great diversity of so far uncultivated spirochetes [11]. In a study of a dentoalveolar abscess with bacteria-specific PCR primers, a number of rDNA sequences were amplified which were not found among the cultivated bacteria from the same abscess sample [12]. In this study we developed primers for PCR to retrieve and sequence archaeal small subunit (SSU) rDNA from subgingival plaque samples. The sequences were subjected to phylogenetic analysis.

2. Materials and methods

2.1. Sampling of subgingival dental plaque and DNA extraction

Subgingival plaques of 48 periodontitis patients were examined. Thirty seven patients (27 female, 10 male) had adult periodontitis, eight (four female, four male) had rapidly progressing periodontitis, one had localized juvenile periodontitis, one had refractory periodontitis and one was an epilepsy patient with periodontitis. The mean age was 44 (range: 16–74) and the mean of the mean pocket depth was 6.8 mm (range: 5–12 mm). None of the patients received antibiotic treatment during a 3-month period pri-

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or to sampling. All plaque samples from each patient were pooled and suspended in 2.5 ml anaerobe transport medium containing (in g l^{-1}) NH_4Cl, 1.0; $MgCl_2$, 1.0; $CaCl_2$, 1.0; resazurin, 0.001; $NaHCO_3$, 2.5; $K_2HPO_4 \cdot 3H_2O$, 0.2; $KH_2PO_4 \cdot 3H_2O$, 0.2; ascorbate, 0.1; L-cysteine·HCl, 0.5 and stored at −20°C. Before DNA extraction 1 ml of thawed suspension was centrifuged at $10\,000 \times g$ at 4°C for 3 min, resuspended and washed in 50 mM Na-acetate/10 mM EDTA pH 5.1. Lysozyme at a final concentration of 1 mg ml^{-1} was added, followed by an incubation at 37°C for 30 min. Proteinase K (0.5 mg ml^{-1}) and sodium dodecyl sulfate (0.5%) were then added and incubation was continued at 50°C for 15 min. Nucleic acids were then extracted by phenol/chloroform treatment and purified by ethanol precipitation. DNA was suspended in 50 μl 10 mM Tris–HCl pH 8.0/1 mM EDTA and the concentration was estimated spectrophotometrically.

Strains of the human methanogens *M. oralis* DSM 7256 [9], an intestinal and a fecal human isolate of *Methanobrevibacter smithii*, DSM 2374 and DSM 2375 [3], respectively, were from the DSM (Braunschweig, Germany) and cultured in media as described [1]. DNA was extracted as described above.

2.2. Selection of PCR primers, PCR amplification, and Southern hybridization

Aligned SSU rRNA sequences were from the Ribosomal Database Project (RDP) [13] and analyzed to select primers specific for archaeal SSU rDNA. The forward primer 300fEyAr 5′-AGC(A/G)(A/G)GAGCCCGGAGATGG-3′ targets at SSU rDNA positions 300–318 (corresponding positions of the *Escherichia coli* SSU rDNA sequence) and the reverse primer 954rEyAr 5′-CGGCGTTGA(A/G)TCCAATTAAAC-3′ at positions 954–934.

Amplification was performed in a Perkin Elmer thermocycler 2400 employing the hot start technique with AmpliWax PCR Gem 100 wax beads. The lower phase (25 μl volume) contained PCR buffer-L (final concentrations of 10 mM Tris–HCl pH 8.85; 5 mM $(NH_4)_2SO_4$; 3 mM $MgSO_4$), primers at 0.4 μM each, dNTP at 0.4 mM each and between 10 ng and 1 μg of template DNA. The upper phase (75 μl volume) contained PCR buffer-U (final concentrations of 10 mM Tris–HCl pH 8.85; 5 mM $(NH_4)_2SO_4$; 3 mM $MgSO_4$; 25 mM KCl) and 2 U of *Pwo* DNA polymerase. The temperature profile was set to pre-PCR denaturation at 95°C for 1 min, 35 cycles of denaturation at 95°C for 15 s, annealing at 64°C for 30 s, extension at 72°C for 15 s, and post-PCR extension at 72°C for 7 min. PCR products were detected after electrophoresis in NuSieve 3:1 agarose gels and ethidium bromide staining. Reamplification of 1 μl PCR product was run for 30 cycles instead of 35 cycles under the conditions described above.

For size fractionating of PCR products agarose blocks containing dsDNA of approximately 0.5–0.7-kb size were excised from a gel and the DNA subsequently extracted with the QIAEX kit. Amplification of 1/20 volume of the purified size-fractionated PCR products was done for 25 cycles at the conditions described above.

Agarose gels containing 10 μl electrophoresed PCR product were blotted to nylon membranes (Biodyne A Pall). Oligonucleotide ARCH915 [14] was labelled with digoxigenin at the 5′-end. Hybridization procedures and conditions were used as described [15].

2.3. Cloning, sequencing, and analysis of PCR products

PCR products were purified on a spin column (PCR Purification kit, Qiagen) and ligated into the *Sma*I site of pBluescript II SK(−). The ligation mixture was used to transform *E. coli* XL1-Blue MRF′ by electroporation. Plasmid DNA was extracted from *E. coli* transformants by alkaline lysis and further purified on Qiagen-tip 20 columns for sequencing with the Ready Reaction Dye Terminator Cycle Sequencing kit with AmpliTaq FS. Oligonucleotides with T3 and T7 primer sequences or primer 954rEyAr (for the direct sequencing of PCR products) were used as sequencing primers. Fragments were analyzed on an ABI 373 automated sequencer.

Homologous positions were aligned manually with the 'SeqEdit' program from the RDP [13]. All 599 characters could be aligned unambiguously to those of *M. smithii* and were compared with relevant *Methanobrevibacter* 16S rDNA sequences. Phylogenetic analysis was done with a beta version of PAUP V4.0 [16]. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor [17]. Phylogenetic trees were constructed employing the neighbor-joining method. The topology of this tree obtained after 1000 bootstraps was compared to trees after 1000 bootstraps of branch-and-bound searches using the maximum-parsimony criterium and after 1000 bootstraps of heuristic searches under the maximum-likelihood criterium.

3. Results and discussion

3.1. Amplification of archaeal rDNA from subgingival plaque

DNAs isolated from authentic cultures of *M. smithii* (DSM 2375) and *M. oralis* (DSM 7256) were used as templates to calibrate PCR conditions. Amplification products were detectable from reactions containing at least 100 fg of *Methanobrevibacter* DNA which corresponds to the DNA content of approximately 50 cells. Nucleic acids extracted from 1 ml of pooled plaque samples yielded between 1.5 and 7.5 μg DNA. 10 ng, 100 ng, and 1 μg of plaque DNA from each sample was subjected to amplification with primers 300fEyAr and 954rEyAr. Out of the

Table 1
Human *Methanobrevibacter* rRNA signatures

Position[a]	Composition in		
	Phylotype 1 and *M. oralis*	Phylotype 2 and *M. smithii*	Phylotype 3
350	C	U	U
543	subtype I = C subtype II = U	C	C
557	G	G	A
560	A	U	U
615:625[b]	U:A	C:G	U:A
658:747[b]	U:A	U:A	C:G
737	A	G	A
770:809[b]	U:A	C:G	U:A
838	A	G	G
850	U	C	C

[a] *E. coli* positions.
[b] Base pairing is according to the secondary structure of the closely related *M. formicicum* SSU rRNA available through the RDP [13].

48 plaque samples tested, 13 yielded a PCR product of 0.65-kb length visible in agarose gels and 12 of these samples (25%) were shown to contain archaeal rDNA by hybridization.

In view of the pronounced complexity of subgingival dental plaque and in analogy to other plaque bacteria, whose frequency is typically in the range of <0.1 to a few % [10], we suspected that the detected *Archaea* occur also in low proportions. Therefore, the 36 samples that did not yield visible quantities of archaeal rDNA were reamplified with the same primer pair in order to assess the genetic diversity of oral methanogens in dental plaque. The ARCH915 probe hybridized to 14 of 16 bands obtained by this reamplification. Additionally, to avoid possible competition by templates smaller than 0.65 kb during reamplification, the original PCR products of the 22 samples that did not reveal archaeal rDNA after amplification nor after reamplification were size-fractionated by gel electrophoresis. DNA of approximately 0.5–0.7 kb was excised and purified from agarose gels and reamplified. Fourteen out of the 22 samples yielded an amplification product of approximately 0.65 kb and the ARCH915 probe hybridized to 11 of these 14 samples.

In summary, archaeal rDNA was detected in 37 (77%) out of 48 subgingival plaque samples. No statistically significant differences were observed with respect to clinical parameters between patients carrying plaque containing or lacking archaea.

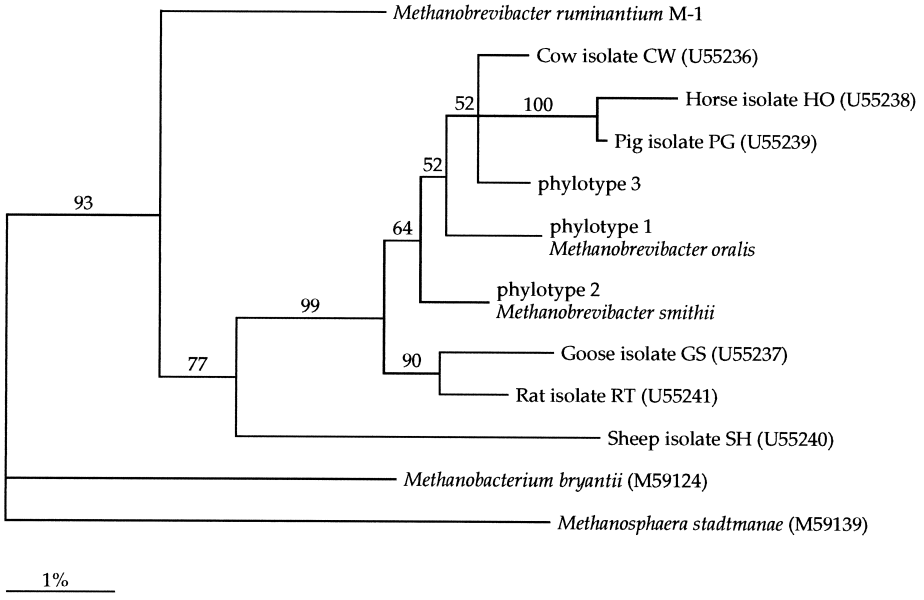

Fig. 1. Unrooted phylogenetic tree of *Methanobacteriaceae* inferred from partial SSU rDNA sequences [4,13]. Accession numbers are in parentheses. The 16S rDNA sequence of *M. ruminantium* was obtained from the RDP (no accession number available). Bootstrap values are indicated above the nodes. The scale bar represents 1% difference in nucleotide sequence. The partial SSU rDNA sequences from *E. coli* positions 319–953 of phylotype 1 (corresponding to *M. oralis* DSM 7256), of phylotype 2 (corresponding to *M. smithii* DSM 2375), and of phylotype 3 have been submitted to EMBL database (accession numbers AJ001709, AJ001710 and AJ001711, respectively).

3.2. Sequencing of amplified rDNA and phylogenetic relationships

Parts of the SSU rDNA sequence from two *M. smithii* strains, DSM 2375 and DSM 2374 [3], and of *M. oralis* DSM 7256 [9] were determined and used as a reference for the amplified subgingival DNA sequences.

Twenty one PCR products were arbitrarily selected from the 37 hybridization-positive amplicons for sequence analysis. DNA from 1–4 recombinant plasmids from each of 19 samples as well as 11 and 14 cloned DNAs from two samples were sequenced. Both strands of a total of 55 recombinant clones were analyzed. Forty six clones from 18 samples contained archaeal rDNA only, whereas in other samples clones with human genomic or, in one case, with bacterial DNA were also detected.

Three different SSU rDNA sequences were amplified from the plaque samples (Table 1). One group (phylotype 1) of 33 PCR clones derived from 16 plaque samples corresponded to *M. oralis*. Within this group, two sequence types were distinguished at *E. coli* position 543. The sequences from 10 plaque samples contained a cytosine, while the others and *M. oralis* DSM 7256 had a uracil at this position. A second group (phylotype 2) of 12 PCR clones from two samples corresponded to both *M. smithii* strains. The *M. oralis* and *M. smithii* groups differed in nine positions as shown in Table 1. The third group (phylotype 3) consisted of one sequence. This sequence had *M. oralis* signatures at five positions and *M. smithii* signatures at four positions and deviated in an additional three positions from both, the *M. oralis* and *M. smithii* sequences (Table 1). It is conceivable that this sequence represents a chimeric molecule [18]. However, this is rather unlikely since this molecule would have had suffered two crossovers and three point mutations as it exhibits three signatures not shared with phylotype 1 or 2. The phylotypes were checked by comparing the base pairing in helices according to the secondary structure of the closely related *Methanobacterium formicicum* SSU rDNA [13]. Compensatory nucleotide substitutions were found in three stem regions, three of the signature nucleotides were in a loop, and at four positions the nucleotide changes resulted in a U-G pairing. These arguments support the existence of phylotype 3.

From the 14 cloned rDNAs obtained from one plaque sample, three displayed *M. oralis* signatures. One of these exhibited three differences from *M. oralis*, two of which corresponded to *M. smithii* signatures. Among the 11 *M. smithii* sequences amplified from this sample, four differed in a single position. One of the latter exhibited a *M. oralis* signature.

The partial sequences of the SSU rDNA were aligned to those of *M. smithii* and analyzed together with other sequences of the *Methanobrevibacter* of human and animal intestinal tract origin by phylogenetic inference. The topologies of the phylogenetic trees constructed by three different methods were in accordance. Fig. 1 shows a dendrogram for the *Methanobacteriaceae* partial rRNA gene sequences. Bootstrap analysis supported the formation of a distinct subgroup of *Methanobrevibacter* species from human and animal sources within the *Methanobacteriaceae*.

The genetic diversity of oral methanogens is quite low and two species, *M. oralis* and *M. smithii*, have been cultivated earlier from human plaque and from human feces. However, a low diversity is not untypical for methanogens and other *Archaea* [19]. This low diversity offered the possibility to identify DNA by sequencing the PCR products of archaeal SSU rDNA from subgingival plaque directly without a cloning step. One strand of 34 of the 37 PCR products to which probe ARCH915 hybridized was sequenced using the reverse PCR primer as sequencing primer. PCR products from 31 patients contained signatures of only one phylotype: 28 phylotype 1 (82%), one phylotype 2 (3%), and two phylotype 3 (6%). The PCR products from three samples (9%) contained both, phylotype 1 and phylotype 2 signatures overlapping each other at each of the nine positions.

Methanogens are thought to remove the metabolic end products of other plaque bacteria which they need for growth (e.g. H_2 and CO_2 or formate). In analogy to their role in anaerobic degradation of waste methanogens could increase total microbial activity and thereby contribute directly or indirectly to tissue damage. To gain insight into the ecophysiology of subgingival dental plaque and to provide new clues for understanding, diagnosis, and therapy of periodontal plaque, other rRNA-based methods including rRNA libraries or quantitative PCR assays could be developed to determine the proportion of methanogens in the subgingival dental plaque flora. Additionally, the prevalence of methanogens in different patient categories can be determined using the established ARCH915 hybridization probe or PCR primers with a broad specificity for *Archaea*.

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